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Developmental and TGF-β-mediated regulation of Ank mRNA expression in cartilage and bone

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Summary

Objectives: Ank encodes a transmembrane protein that is involved in pyrophosphate (PPi) transport and mutations in the *Ank* gene have been associated with pathological mineralization in cartilage and bone. To understand how Ank works in normal skeletal development it is also important to know which cells within the developing skeleton express Ank. To this end, we examined the expression pattern of Ank mRNA during mouse embryonic development as well as in mouse hind limb joints with emphasis on the period when articular cartilage forms. Since it was previously shown that TGF-β regulates PPi transport in cells in culture, we also tested the hypothesis that TGF-β regulates Ank expression.

Methods: The localization of Ank mRNA was determined by radioactive *in situ* hybridization in E15.5 and E17.5 mouse embryos as well as in 1 and 3 week post-natal mice. Ank expression was compared to that of other cartilage markers. *In situ* hybridization and semi-quantitative RT-PCR were used to determine the effects of TGF-β on Ank expression in metatarsal organ cultures.

Results: Ank expression was detected at high levels at sites of both endochondral and intramembranous bone development. In endochondral bones, expression was detected in a subset of hypertrophic cells at ossification centers. Expression was also detected in osteogenic/chondrogenic cells of the perichondrium/periosteum lining the metaphysis, an area associated with the formation and extension of the bone collar. High levels of expression were also detected in non-mineralized tissues of the skeletal system including tendons and the superficial layer of the articular cartilage. Treatment with TGF-β resulted in an approximately four-fold induction of Ank mRNA in prehypertrophic chondrocytes and perichondrium of metatarsal cultures.

Conclusions: The expression pattern of Ank suggests an important role both in inhibiting and regulating mineralization in the developing skeletal system. In addition, TGF-β1 is able to mediate Ank mRNA expression in chondrocytes suggesting a possible role for TGF-β and Ank in the regulation of normal mineralization.

Key words: *Ank*, Transforming growth factor-beta, Pyrophosphate, Mineralization.

Introduction

Bones of the vertebrate skeleton are formed by either intramembranous or endochondral ossification (reviewed in Ref. 1). During intramembranous ossification mesenchymal cells differentiate directly into preosteoblasts and osteoblasts. Mineralization of the matrix follows, first in an irregular pattern leading to the formation of woven bone which is subsequently remodeled and replaced with lamellar bone. Bones formed by intramembranous ossification include the flat bones of the head and the clavicle. Most of the bones in the body, including the long bones of the appendicular skeleton form by the process of endochondral ossification. Endochondral bones are derived form a cartilage model[1–3]. During embryonic development mesenchymal cells condense and differentiate into chondroblasts forming the initial shape of the skeletal element. Cells then undergo a complex program of proliferation, differentiation, and hypertrophy resulting in a matrix that can be mineralized and replaced with bone. Recapitulation of this process in the growth plate allows for longitudinal growth of bones after birth. After the initial shape of the skeletal element is formed, cells from the perichondrium (which at this point becomes the periosteum) at the center of the element differentiate to form a bone collar[4]. The bone collar marks the ossification center of the bones and precedes vascular invasion and mineralization of the cartilage. Formation and extension of the bone collar can be regarded as intramembranous ossification. Appositional growth of the bone also occurs from the perichondrium/periosteum.

Although most of the cartilage model will be replaced with bone there are several sites where mature cartilage persists throughout life, for example the articular cartilage on joint surfaces. The formation of the articular cartilage in mouse is similar to that described for human[5], rabbit[6], and opossum[7]. The femoral and tibial epiphysis of the newborn mouse are composed entirely of cartilage. At 7–10 days after birth, a secondary ossification center forms in the epiphysis, delineating the epiphyseal growth plate cartilage from the future articular cartilage. By 2 weeks of age, the articular cartilage begins to show some of the organizational characteristics of adult articular cartilage; however, endochondral bone formation still occurs in the deepest layers of the cartilage next to the ossification center. At 3 weeks of age, only a few hypertrophic cells are still visible

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in the deep zones of the articular cartilage as the formation of the bone and marrow cavity in the epiphysis nears completion. After 4 weeks of age three main zones of adult articular cartilage are visible and there is a sharp demarcation between the cartilage and the subchondral bone. Surprisingly little is known about factors that prevent articular cartilage from becoming mineralized and replaced with bone.

Progressive ankylosis in mouse (*ank/ank*) is an autosomal recessive form of joint destruction characterized by pathological mineralization in the articular surfaces and synovium[8]. The *Ank* locus was recently shown to encode a multipass transmembrane protein, called Ank, that regulates pyrophosphate (PPi) transport[9]. Expression of Ank in cultured cells resulted in increased elaboration of extracellular PPi that was blocked by the addition of probenecid, an inhibitor of anion transport[9]. Available data is consistent with the possibiltiy that Ank may act a regulator of an anion channel rather than exist as the channel itself (reviewed in Ref. 10;[9]). PPi has been recognized as a direct and potent inhibitor of mineralization in connective tissue matrix (reviewed in Ref. 10). Articular cartilage and other non-mineralized skeletal tissues including the meniscus have a high level of extracellular PPi relative to mineralized tissues so it has been proposed that Ank acts to prevent mineralization of articular cartilage by regulating the level of PPi in the matrix[9,10]. Ank mRNA has been detected by non-radioactive *in situ* hybridization in cells at the articular surface in forelimbs of mouse embryos at 16 days of gestation[9]. Localization of Ank mRNA in other developing bones or in the post-natal period when the articular cartilage forms was not reported.

Members of the TGF-β superfamily are secreted signaling proteins that regulate many aspects of development including growth, differentiation, and mineralization in skeletal tissue[11–13]. Transgenic mice expressing a dominant-negative mutation of the TGF-β type II receptor develop an osteoarthritis (OA)-like syndrome that includes progressive loss of joint mobility, cartilage and bone growth in the joint space an synovium, and formation of osteophytes on the joint surface suggesting a role for TGF-β in formation and maintenance of the articular cartilage[14]. Furthermore, mineralization is inhibited by TGF-β1 in embryonic metatarsal bones grown in organ culture[15,16]. TGF-β has been shown to stimulate PPi elaboration from articular chondrocytes in culture[17]. This effect was blocked by probenecid suggesting that TGF-β acted through an anion transporter[18]. It is therefore possible that TGF-β may at least in part regulate mineralization through the regulator of PPi transport, Ank. In this report we characterize the expression pattern of Ank in embryonic and post-natal skeletal development and test the hypothesis that TGF-β regulates Ank mRNA expression in chondrocytes.

Materials and methods

PCR CLONING ANK INTO PBLUESCRIPT VECTOR

Ank was cloned into the pGEM-T easy vector using the TA cloning kit from Promega. Ank cDNA was amplified from cDNA made from mouse brain RNA using the following primers: AnkR CAGGAATTTAGTGAACTGTGTCC and AnkF ACAGAGGAGGTCACAGACATC amplifying nucleotides 1462 to 2593 of the Ank gene (GenBank AF274752;[9]). The identity of the clone was verified by sequencing. In addition, two bands of the expected size ([9]; 3.5 and 4.0 Kb) were observed in Northerns blots of RNA from epiphyseal cartilage (data not shown)

IN SITU HYBRIDIZATION

In situ hybridization of mouse skeletal tissue has been described[14,19]. Joints from at least three separate mice were studied and representative images from one mouse are shown. The following probes were used in this study: ank (described above); collagen type IIA (pSOK10;[20]); collagen type IIB (pEL111;[21]); collagen type X (pSAm10h;[22]); decorin (pmDcncDNA ex2 to ex7; gift from Dr Vogel, University of Ulm, Germany;[23]).

METATARSAL CULTURE

Metatarsal culture and treatment with TGF-β1 has been described[15,16].

RT-PCR

RNA isolation and RT-PCR analysis have been described in[16]. Primers used for Ank are described above. Primers for glyceraldehyde-3-phosphate dehydrogenase were: 3′ CAT GTA GGC CAT GAG GTC CAC CAC; 5′ TGA AGG TCG GTG TGA ACG GAT TTG GC.

Results

EXPRESSION PATTERN OF ANK MRNA IN MOUSE EMBRYOS

To begin to understand how *Ank* regulates normal skeletal development, the expression pattern of Ank mRNA during mouse embryonic development was determined using radioactive *in situ* hybridization. Sagittal sections from mouse embryos at 15.5 and 17.5 days of gestation were hybridized to an ^{35}S-labeled Ank riboprobe. The highest levels of Ank mRNA were detected spatially and temporally at known sites of bone development and mineralization (Fig. 1). These sites included both developing intramembranous and endochondral bones.

In the head [Fig. 1(a),(b)], hybridization to the Ank riboprobe was detected in the mandible, maxilla, and frontal bone (intramembranous) as well as ossification centers of the basioccipital and basisphenoid bones (endochondral). Hybridization was detected in the clavicle, another intramembranous type bone. Within the intramembranous bones, Ank mRNA was localized to mesenchymal cells within the condensation of the bone anlagen.

Ank mRNA was detected in ossification centers long of bones including the humerus, tibia, fibula, and femur which are shown in Fig. 1(a),(c). Ank mRNA was not observed in metatarsal bones at 15.5 days of gestation before the formation of the ossification center. By embryonic day 17.5, Ank mRNA was visible in hypertrophic chondrocytes at the site of the newly formed ossification center [Fig. 1(d)]. In bones in which the ossification center was more developed, Ank mRNA was detected in cells lining the inner layer of the perichondrium/periosteum at the diaphysis near the hypertrophic zone of cartilage [Fig. 1(c)]. This is an area where the bone collar is forming to extend the central part of the ossification center. In addition, hypertrophic chondrocytes next to these ossification centers expressed Ank mRNA [Fig. 1(c)]. Similarly, in the ribs, positive cells were lined up

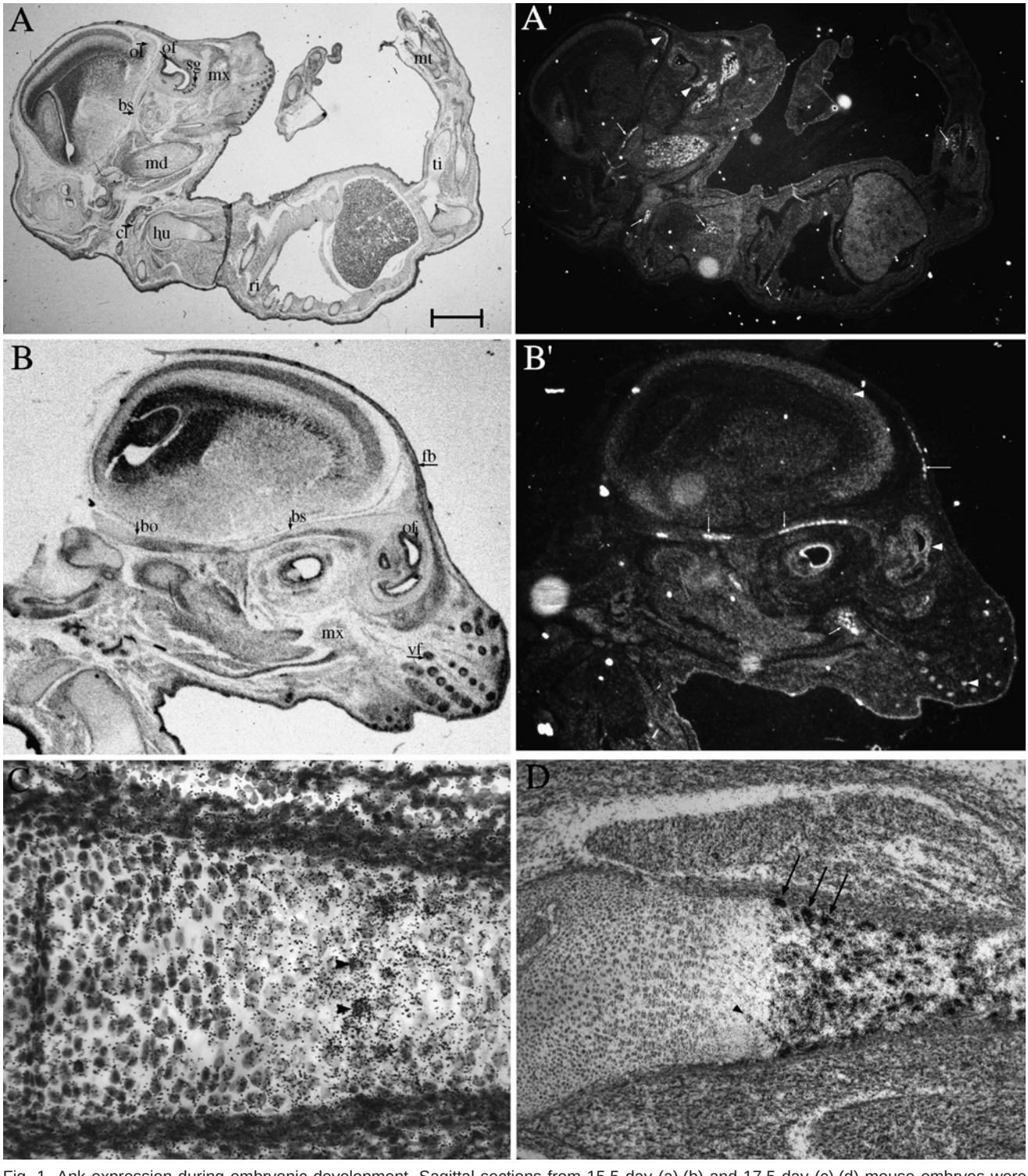

Fig. 1. Ank expression during embryonic development. Sagittal sections from 15.5 day (a),(b) and 17.5 day (c),(d) mouse embryos were hybridized to ^{35}S-labeled antisense riboprobe for Ank. Bright field and dark field images are shown in (a) and (b). Hybridization is seen as bright grains in the dark field image. In (c) and (d) the bright field image was inverted and superimposed on the bright field image using photoshop software. Hybridization is seen as black grains on the image. Hybridization at skeletal sites is denoted with a small white arrow in (a′) and (b′). Hybridization to non-skeletal sites is marked with a white arrowhead. Black arrowheads denote positive hypertrophic cells in the 17.5 day metatarsal (c) and femur (d). Positive cells lining the inner layer of the perichondrium are marked with black arrows in (d). Hybridization was not detected to a nonsense riboprobe (data not shown). bo, basioccipital bone; bs basisphenoid bone; cl, clavicle; fb, frontal bone; hu, humerus; md, mandible; mt, metatarsal; mx, maxilla; of olfactory epithelium; ol, olfactory lobe; ri, rib; sg, serous gland; ti, tibia; vf vibrissae follicle. Scale bar A, 1.4 mm; B, 1.0 mm; C, 100 μm, D, 50 μm.

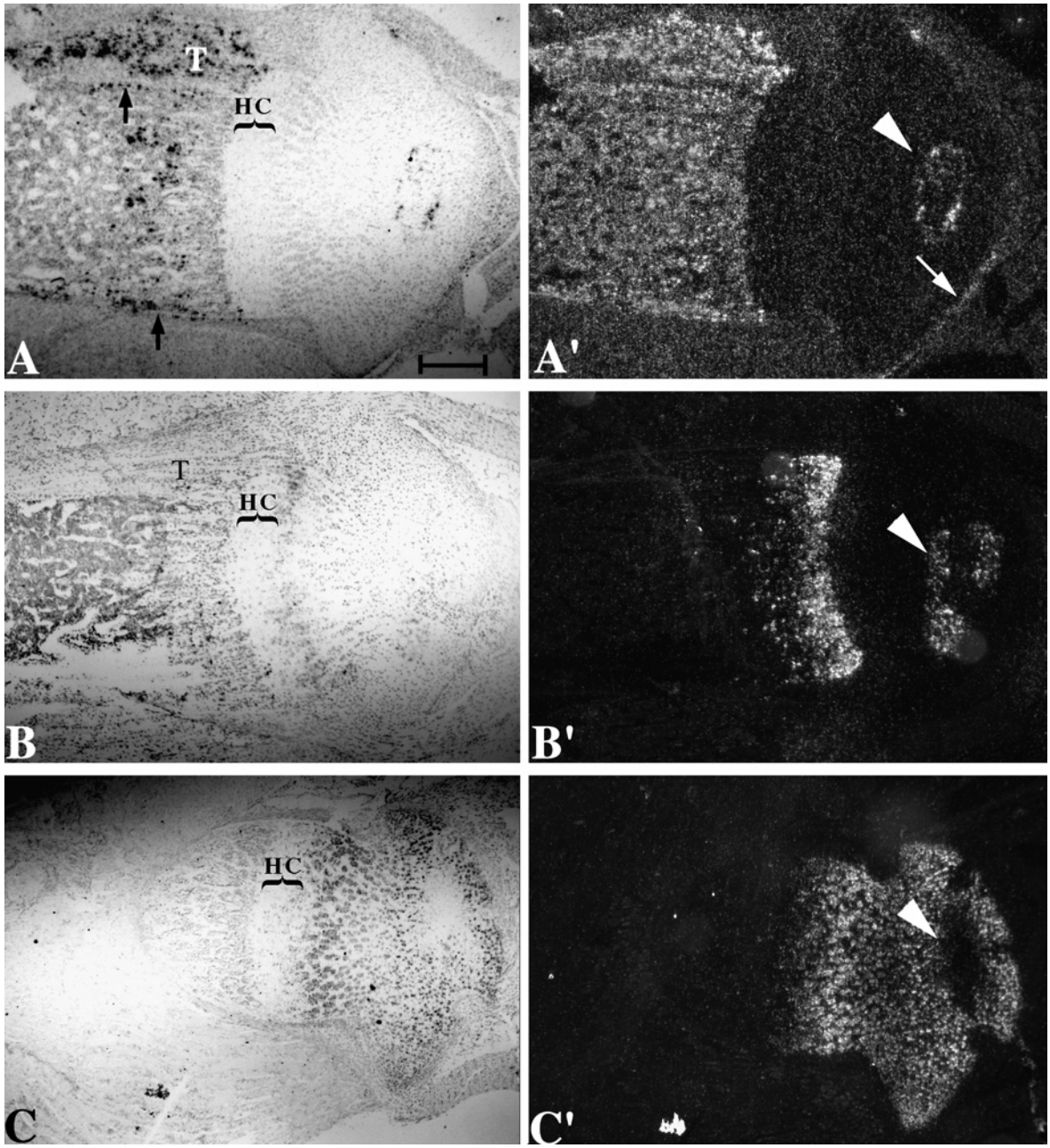

Fig. 2. Ank mRNA localization at 1week. Sections of tibia from 1-week-old mice were hybridized to the indicated ^{35}S-labeled riboprobes. Bright field and dark field images are shown. Hybridization is detected as white grains on the dark field image. Ank mRNA was detected in the secondary ossification center [(a′), white arrowhead], the superficial layer of cells of the future articular cartilage [(a′), white arrow], cells of the inner perichondrium [(a) black arrow] and in the tendon [(a), T]. Hybridization to the Collagen type X probe was detected in hypertrophic cartilage (HC) of the secondary ossification center [(b′), white arrow head] and growth plate [(b), brackets]. Collagen type IIBm RNA was detected in all cartilage except hypertrophic cartilage (HC) in the secondary ossification center [(c′), white arrowhead] and in the growth plate [(c), brackets]. Hybridization was not detected to a sense Collagen type X probe (not shown). Scale bar=250 μm.

along the inner edge of the perichondrium/periosteum in areas corresponding to the location of bone collar formation near sections of histologically hypertrophic cartilage [Fig. 1(a)]. Ank mRNA was also detected in stromal fibroblast invading the future marrow cavity [Fig. 1(c)].

Ank was not restricted to skeletal tissues [Fig. 1(a),(b)]. For example, hybridization was observed in vibrissae follicles, the future cerebral cortex, olfactory epithelium and associated serous glands, and cells within the olfactory lobe in the brain.

EXPRESSION PATTERN OF ANK MRNA IN POST-NATAL BONE DEVELOPMENT

Since mutations in *Ank* result in joint destruction[9], we next focused our investigation on Ank expression during normal articular cartilage development. Ank mRNA was localized in tibia from 1- and 3-week post-natal mice in relation to several cartilage markers (Fig. 2; see Fig. 4). In 1 week post-natal mice, Ank mRNA was localized to hypertrophic cells near the secondary ossification center

[Fig. 2(a), arrowhead]; Fig. 3(a) as well as hypertrophic cells at the transition of the growth plate and trabecular bone [Figs 2(a), 3(b)]. Ank mRNA was also detected in osteocytes embedded within the trabecular bone matrix near the end of the growth plate [Fig. 2(a)]. Ank mRNA was not detected in cells lining the trabecular bone. Collagen type X is a marker for hypertrophic cartilage and is not normally detected in articular cartilage from adult mice[22]. Collagen type X was detected in the area of the secondary ossification center, where hypertrophic differentiation occurs [Fig. 2(b), arrowhead] and in hypertrophic chondrocytes of the growth plate [Fig. 2(b), brackets]. The Ank expression domain overlapped with only a small part of the Collagen type X domain. Only a subset of histologically hypertrophic cells in the secondary ossification center and in the deepest layers of the growth plate expressed Ank mRNA [Figs 2; 3(a),(b)]. Collagen type IIB is specific for cartilage and is its major collagen species[24]. Collagen type IIB was excluded from the secondary ossification center where Collagen type X and Ank were observed [Fig. 2(c), arrowhead].

In hindlimb joints from 3-week post-natal mice, Ank mRNA continued to be detected in a subset of hypertrophic cells at the transition between the articular cartilage and the secondary ossification center [Fig. 4(a)]. Collagen type X was still detected in this deep level of the developing articular cartilage [Fig. 4(b)], presumably from normal development occurring in the secondary ossification center. Again, the expression domain of Collagen type IIB and Ank did not overlap [Fig. 4(c)]. At this age, the formation of the secondary ossification center is almost complete, demarcating the separation of the articular cartilage from the rest of the bone. Fewer cells at the edge of the ossification center expressed Ank or Type X collagen than at 1 week post-partum when differentiation and mineralization occur rapidly.

Ank mRNA was localized to the osteogenic/chondrogenic layer of the periosteum/perichondrium lining the metaphysis at the junction of the growth plate and the trabecular bone in mice at 1 week and 3 weeks post-partum [Figs 2(a), 3(b), and 4(a)]. In addition, osteoblasts within the osteoid that were lined up along the periosteum expressed Ank mRNA [Figs 2(a), 3(b) and 4(a)]. This is an area where appositional growth occurs and cortical bone will form to extend the bone collar that was formed earlier in development. Ank mRNA was excluded from or was expressed at very low levels in the cortical bone itself and the periosteum of the diaphysis.

Ank was also expressed in skeletal tissues that do not undergo mineralization. High levels of Ank were detected in tendon cells [Fig. 2(a)] and, as previously reported for embryonic joints[9], Ank mRNA was observed in the superficial layer of the articular cartilage [Fig. 2(a), arrow; Figs 3(c) and 4(a)]. Articular cartilage remains unmineralized throughout life and it was proposed that Ank expression in the superficial layer of the articular cartilage and the resulting levels of PPi in the matrix prevent mineralization. The expression pattern of Ank suggests that the gene product

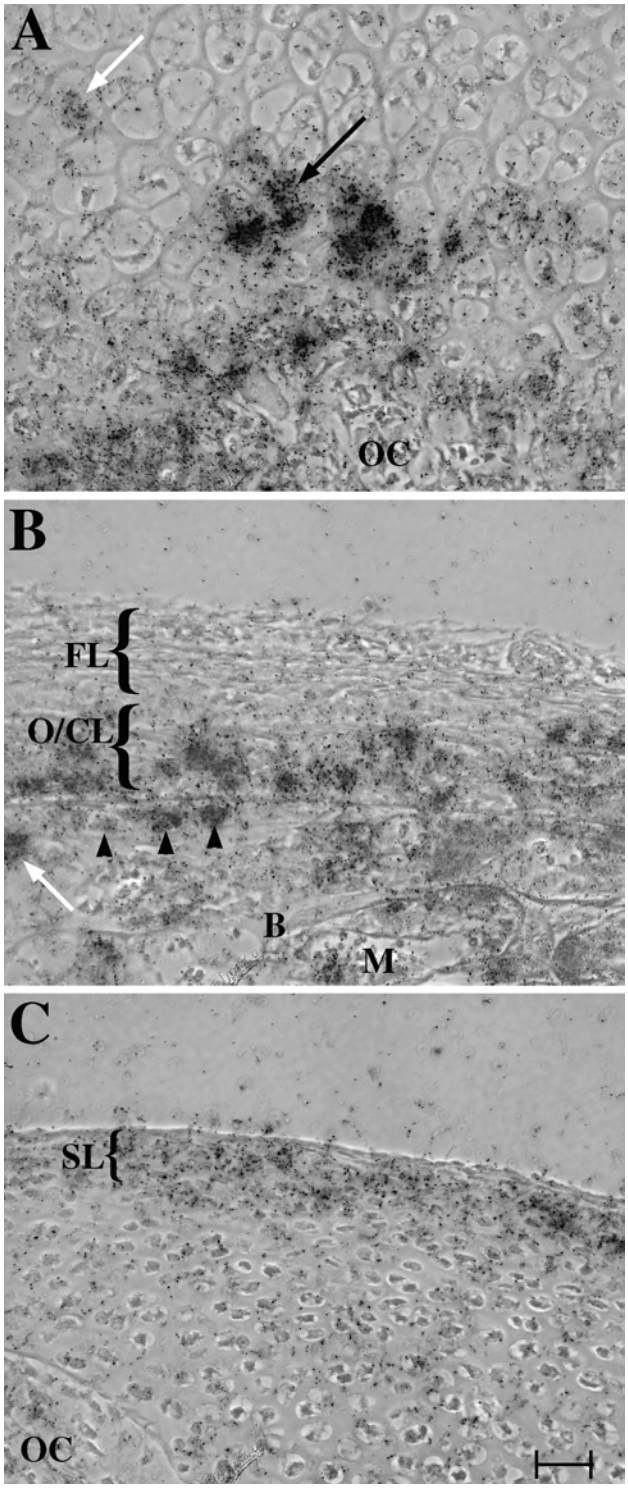

Fig. 3. Ank mRNA localization at 1 week, high magnification. Sections from 1 week hind limb joints were hybridized to a ^{35}S-labeled riboprobe to Ank. Phase contrast pictures are shown. Hybridization is seen as dark grains over specific cells. (a) Hybridization to Ank riboprobe in hypertrophic cells near the secondary ossification center (OC). Cells proximal to the ossification center express higher levels of Ank (black arrows) relative to hypertrophic cells that are more distal (white arrow). (b) An area of the perichondrium at the junction of the growth plate and trabecular bone of is shown. Ank mRNA is localized to cells within the osteogenic/chondrogenic layer of the perichondrium (O/CL) but is not detected in the fibrous layer (FL). Expression is also detected in cells in the osteoid layer underlying the periosteum (arrowhead) as well as hypertrophic cells at the transition between the growth plate and trabecular bone (white arrows). Marrow cavity (M), trabecular bone (B). (c) Ank expression in the superficial cells (SL) of the future articular cartilage. Scale bar=25 μm.

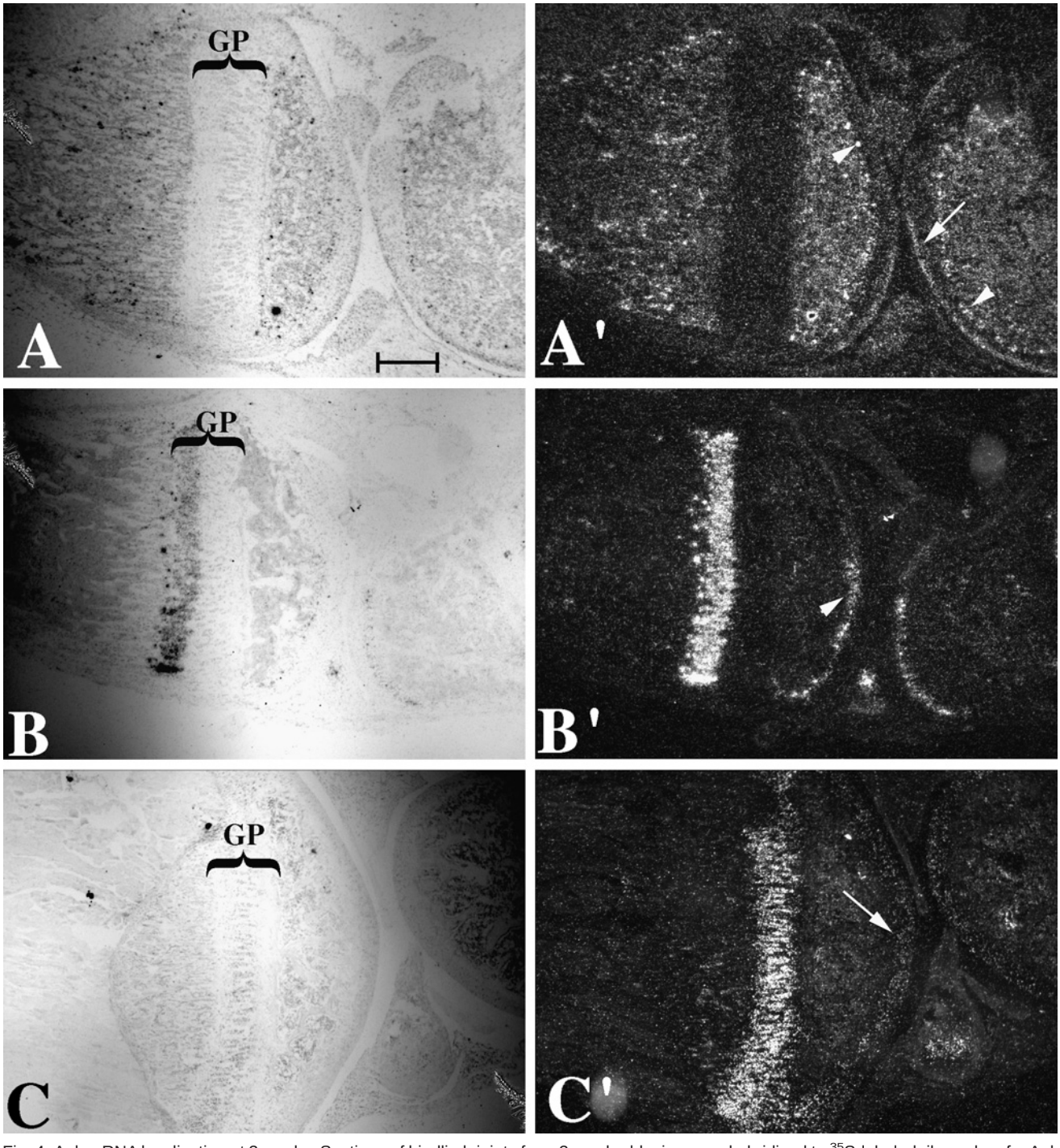

Fig. 4. Ank mRNA localization at 3 weeks. Sections of hindlimb joints from 3-week-old mice were hybridized to ^{35}S-labeled riboprobes for Ank (a), Collagen type X (b), and Collagen type IIB (c). Bright field and dark field images are shown. Hybridization is detected as bright white grains on the dark field image. Hypertrophic cells between articular cartilage and subchondral bone are indicated by arrowheads. Expression of Ank in the superficial zone of the articular cartilage is indicated by an arrow. Hybridization was not detected to a sense type X collagen probe (not shown). Scale bar=250 μm.

may have a role in both preventing mineralization in non-mineralized skeletal tissue and in regulating normal mineralization at other skeletal sites.

REGULATION OF ANK MRNA EXPRESSION BY TGF-β

Since it was previously shown that TGF-β regulates PPi transport in cells in culture[17,18], we tested the hypothesis that TGF-β regulates expression of Ank in metatarsal bones grown in organ culture. Metatarsal bones from 15.5 day old mouse embryos were isolated and grown in a chemically defined medium. Cultures were either treated with 10 ng TGF-β1/ml or left untreated for 5 days. Previously, it was shown that treatment with TGF-β1 inhibits its mineralization in the cultures through a signaling pathway distinct from those that regulate growth and

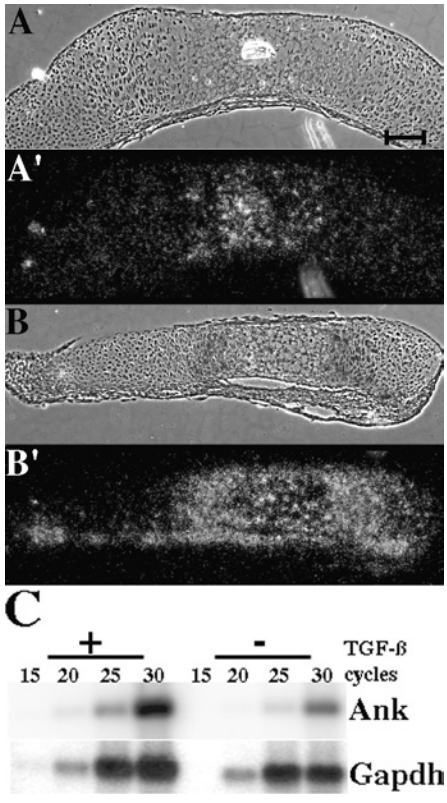

Fig. 5. TGF-β mediated expression of Ank. Sections from untreated (A) and TGF-β1 treated (10 ng/ml; B) embryonic mouse metatarsal bones grown in organ culture were hybridized to a ^{35}S-labeled Ank riboprobe. Phase contrast and dark field images are shown. Hybridization is detected as white grains on the dark field image. In untreated cultures, Ank is detected in a subset of hypertrophic chondrocytes in the center of the bone rudiment (a). In treated cultures, Ank is detected in prehypertrophic and hypertrophic cells as well as cells in the perichondrium (b). Scale bar=50 μm. (C) RT-PCR. The level of Ank mRNA induced by treatment with TGF-β1 was determined using semiquantitative RT-PCR. Embryonic metatarsal bones in cultures were either left untreated (−) or treated (+) with TGF-β1 for 5 days. Ank and Gapdh were amplified for varying cycles. PCR products at 15, 20, 25, and 30 cycles are shown. PCR products were blotted to a nylon membrane and hybridized to ^{32}P-labeled cDNA probes for Ank and Gapdh. Blots were exposed to a Molecular Dynamics Phosporimager screen an band intensities were quantified. The experiment was repeated three times with three separate sets of cultures.

hypertrophic differentiation[15,16]. Ank expression was determined by *in situ* hybridization of sections from untreated bones and bones treated with TGF-β1 [Fig. 5(a),(b)]. In control cultures, Ank was localized to a subset of hypertrophic cells the center of the bone rudiment where mineralization occurs; similar to what is seen *in vivo*. In TGF-β1 treated cultures, Ank mRNA was localized to both prehypertrophic and hypertrophic cartilages as well as the perichondrium, suggesting that TGF-β is sufficient to stimulate expression of Ank in chondrocytes and other cell types.

The level of Ank induction was determined using semiquantitative RT-PCR [Fig. 5(c)]. Ank and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were amplified from cDNA made from RNA extracted from bones that were either untreated or treated with TGF-β1 for 5 days. Gapdh was used to control for the amount and quality of RNA used in each reaction. PCR amplification was performed for a varying number of cycles to determine the linear range for PCR product formation. Ank product formation was linear at 25 and 30 cycles and Gapdh was linear at 20 cycles. PCR products were blotted to nylon membranes and hybridized to radioactively labeled Ank and Gapdh probes. Band intensity was quantified using a phosporimager. Ank results were normalized to that for Gapdh. The experiment was repeated three times with three separate sets of cultures each with approximately 36 bone rudiments for each condition. Treatment with TGF-β1 resulted in a mean 3.64-fold increase in Ank mRNA levels relative to untreated controls (standard deviation=0.2-fold; T-test *P* value=0.000014) indicating that TGF-β can regulate Ank expression in skeletal tissue.

Discussion

The expression pattern of Ank is consistent with the finding that mutations in *Ank* can affect both endochondral and intramembranous bones. Recently, autosomal dominant craniometaphyseal dysplasia (CMD) was shown to be associated with mutations in the human ortholog to the mouse *Ank* gene[25,26]. CMD is characterized by overgrowth and increased mineral density of craniofacial bones as well as abnormal, flared metaphyses (MIM 123000). In the mouse, Ank mRNA was detected in mesenchymal cells of intramembranous bone anlagen during embryonic development. Furthermore, localization of Ank mRNA in hypertrophic cartilage and in the metaphysis where bone collar extension and appositional growth occur is consistent with the flaring observed in CMD.

In contrast to CMD, a nonsense mutation in murine *Ank* results in an autosomal recessive condition known as progressive ankylosis (*ank/ank*). Mice with the *ank/ank* mutation demonstrate many features of arthritis including ectopic calcification in the joints, cartilage erosion and osteophyte formation, as well as fusion of the vertebrae[27]. Synthesis of Ank in the superficial layer of the articular cartilage may account for this phenotype since Ank acts to regulate PPi transport and it has been shown that PPi can inhibit mineralization in articular cartilage[9,10]. Abnormalities in craniofacial bones or metaphyses have not been described in the *ank/ank* mice. It was proposed that the mutations isolated from CMD patients code for either dominant-negative[26] or gain of function[25] mutations as opposed to ablation of Ank expression as observed for the mouse mutation[9]. The phenotypes associated with these mutations and the localization of Ank at sites of mineralization and at skeletal sites that remain unmineralized suggest that the precise level of extracellular PPi must be maintained for proper skeletal development. It has been shown that both reduced levels of PPi and excess accumulation of PPi result in pathological mineralization associated with basic calcium phosphate or calcium pyrophosphate dihydrate crystals respectively[10,28].

Numerous mediators of bone mineralization have also been shown to mediate the generation of PPi[10]. TGF-β was shown to regulate the generation of extracellular PPi in chondrocyte cultures in a probenecid sensitive manner[17,18] and recently it was shown that generation of PPi by Ank is also probenecid sensitive[9]. Here we show that TGF-β stimulates Ank expression in chondrocytes in organ culture. TGF-β-mediated regulation of Ank may account for the probenecid sensitive accumulation of PPi previously observed in response to TGF-β[18]. TGF-β has also been shown to regulate PC-1, a member of the

phosphodiesterase nucleotide pyrophosphate family that generates PPi directly by pyrophosphohydrolysis of the phosphodiesterase I bond in purine and pyrimidine nucleoside triphosphates[29,30]. PC-1 deficient mice have what is called the 'tiptoe walking' phenotype which shares some similarities with the hypermineralization phenotype observed in the *ank/ank* mice[31]. PC-1 is expressed at overlapping and non-overlapping sites with Ank[10]. PC-1 is expressed in chondrocytes within the articular cartilage and in hypertrophic chondrocytes in the calcifying zone of the growth plate. PC-1 is also expressed in osteoblasts and osteocytes as well as in articular and periarticular ligaments. All three TGF-β isoforms are widely expressed in skeletal tissues[19,32–34] and expression overlaps significantly with Ank and PC-1 raising the possibility that TGF-β can regulate the expression or function of these genes *in vivo*.

Previously we generated transgenic mice that express a dominant-negative mutation of the TGF-β type II receptor (DNIIR) in articular cartilage, fibrous layers of the periosteum/perichondrium, synovium, and a subset of cells in the hypertrophic cartilage of the growth plate. DNIIR mice demonstrate a joint phenotype strikingly similar to that of the *ank/ank* mouse[14] including progressive loss of mobility in the joints, bone and cartilage growth in the joint space, osteophyte formation and fusion of the vertebrae. Expression of the DNIIR transgene overlaps with Ank expression only in the superficial layer of the articular cartilage and we were not able to detect any significant alterations in Ank expression in these cells using radioactive *in situ* hybridization (data not shown). It is possible that in addition to regulating expression of Ank mRNA, TGF-β modulates the activity of Ank via post-translational mechanisms. Alternatively, the similarities in the phenotype may be due to alterations in the expressions of other genes associated with cartilage differentiation or mineralization that do not interact directly with *Ank*.

We and others have shown that TGF-β1 can regulate mineralization in embryonic metatarsal organ cultures[15,16]. It was proposed that inhibition of mineralization was independent of TGF-β's effects on growth and hypertrophic differentiation[15]. The effects on mineralization were independent of PTHrP while the effects on hypertrophic differentiation were dependent on the presence of PTHrP[16]. PTHrP 1-173 has been shown to inhibit the elaboration of extracellular PPi in both costal and articular chondrocytes[35,36]. Regulation of extracellular PPi was controlled by the 147–150 KKKK motiff which was also shown to regulate nuclear localization[36]. Ank mRNA was localized only to a subset of hypertrophic cells in the center of untreated metatarsal cultures. In the presence of TGF-β1, Ank mRNA was detected in prehypertrophic chondrocytes and in the perichondrium. In the metatarsal culture experiments (Fig. 5), Ank was induced almost four-fold after treatment with TGF-β1. It is interesting to speculate about a connection between increases in PPi that would result from higher levels of Ank synthesis and inhibition of mineralization mediated by TGF-β1 in this system.

In summary, we have shown that Ank mRNA is expressed in the skeletal system in a pattern that suggest that it regulates development of both mineralized and non-mineralized skeletal tissue. We show that TGF-β can stimulate expression of Ank mRNA and suggest a testable model where TGF-β and Ank act together to regulate mineralization.

Acknowledgments

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